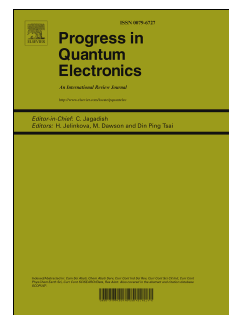


# Accepted Manuscript

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# Laser-induced generation of singlet oxygen and its role in the cerebrovascular physiology

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## Abstract

For over 55 years, laser technology has expanded from laboratory research to widespread fields, for example telecommunication and data storage amongst others. Recently application of lasers in biology and medicine presents itself as one of the emerging areas. In this review, we will outline the recent advances in using lasers for the generation of singlet oxygen, traditionally used to kill tumour cells or induce thrombotic stroke model due to damage vascular effects. Over the last two decade, completely new results on cerebrovascular effects of singlet oxygen generated during photodynamic therapy (PDT) have been shown alongside promising applications for delivery of drugs and nanoparticles into the brain for therapy of brain cancer. Furthermore, a "gold key" has been found to overcome the limitations of PDT, such as low light penetration and high toxicity of photosensitizers, by direct generation of singlet oxygen using quantum-dot laser diodes emitting in the near infrared (NIR) spectral range. It is our motivation to highlight these pioneering results in this review, to improve understanding of the biological role of singlet oxygen and to provide new perspectives for improving clinical application of laser based therapy in further research.

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## 1. Introduction

Singlet molecular oxygen  $^1\text{O}_2$  became the focus of intense laboratory study since seminal papers by Mulliken of 1928. Important treatises were provided by many researchers see for instance reviews [1, 2].

It was understood that  $^1\text{O}_2$  might be a reaction intermediate in dye-sensitized photooxygenation that became a “gold key” in the door of photodynamic therapy (PDT). PDT is a form of therapy that combines a light source and photosensitizing agents (porphyrins, chlorophylls and many other types of photodynamic dyes). The systematically or topically administered photosensitizer is specifically accumulated in diseased tissue. When concentration of photosensitizer is sufficient, it is activated by exposure to light of visible or NIR spectrum appropriated for its effective excitation. The excited photosensitizer interacts with triplet state of molecular oxygen ( $^3\text{O}_2$ ) and produces  $^1\text{O}_2$  resulting the tissue oxidation. The mechanism of  $^1\text{O}_2$  generation is the transition of electrons from the ground state (triplet) in a short-lived ( $\tau_{fl} = 10^{-6}$ – $10^{-9}$  sec) higher-energy electronic state (singlet).

The PDT is a technique developed to treat the ever-increasing global incidence of cancer [1-4]. Current PDT of cancer is based on the photodynamic effect (PDE) with resultant photosensitized cell damage (via apoptotic scenario and endothelial injuries for cancer cells) in the presence of light and oxygen. PDT is usually performed with use of a photosensitizer (PS), which is however absorbed by both healthy and tumour tissues. Due to limitations in particularly: high intrinsic toxicity, prolonged hypersensitivity to intensive light (in case of lung cancer up to 6 weeks), low tissue penetration by activating light (630 nm), low specificity of PSs to cancer types, and a high cost of PSs

administration, in the use of conventional PDT, there is an urgent need for further research and improvements in PDT methods.

Recent development of quantum-dot (QD) laser diodes (LDs) emitting in the near infrared (NIR) spectral range has opened up new venues in low-intensity laser therapy. The QD-LD emission wavelength centred at around 1268 nm coincides well with the NIR absorption band [1, 5] of oxygen molecule. One particular opportunity involves activation of the apoptotic response through direct molecular oxygen photoexcitation. To date the idea of direct  $^1\text{O}_2$  activation has not attracted much attention because direct  $^3\text{O}_2 \rightarrow ^1\text{O}_2$  transition in molecular oxygen is forbidden due to spin-orbital selection rules. However the experimental examinations of solvent effect have redrawn the selection rules governing the intermolecular enhancement [1, 5, 6]. The enhancement of  $^3\text{O}_2 \rightarrow ^1\text{O}_2$  transition was attributed to the fact that the major intensity contribution originates from  $\text{O}_2\text{-O}_2$  bi-molecular collisions, which mix states by an intermolecular exchange interaction, introducing allowed states into previously forbidden transitions. Furthermore, the action spectra of a number of cell cultures, recorded in the spectral range from 310 to 860 nm, and the results demonstrated for low-intensity laser therapy [7, 8] suggest transformation of cell metabolism in response to low power laser excitation in the spectral intervals consistent with absorption bands of molecular oxygen.  $^1\text{O}_2$  formation by direct photoexcitation with 1265 nm in pigment-free aerobic systems [1, 5] and in condensed phase at 77 K with 1064 nm [9] have also been demonstrated. Recently it was demonstrated that 1268 nm laser could induce cancer cell death in PS-free medium [10]. However, it is still unclear whether the laser directly photoactivates molecular oxygen in true bio-systems or whether this is a more complex effect [7, 8, 10]. For the better understanding of these processes, we discuss in this review the mechanisms underlying direct generation of singlet oxygen including experimental data.

Another wide application of vascular effect of PDT is to induce thrombotic stroke model for the studies of neuroprotective effects of anti-stroke pharmacological agents [13-17]. However, there are contradictory results of *in vivo* and *in vitro* studies of thrombotic effect of  $^1\text{O}_2$ , which make challenges to understand true mechanisms of thrombotic stroke and to use this model for the development of new strategies in antithrombotic therapy and effective prevention of stroke [13-21]. To motivate the detailed further studies of mechanism responsible for cerebrovascular effects of  $^1\text{O}_2$ , we describe in this review different effects of  $^1\text{O}_2$  on the process of coagulation *in vivo* and *in vitro* studies.

Over two last decades there were accumulated new promising results in the field of cerebrovascular effects of  $^1\text{O}_2$ . In particular, the PDT-based approaches in modulation of blood-brain barrier (BBB) function and improvement of delivery of drugs, genes, nanoparticles into the brain tissues for the treatment of gliomas [22-25]. However, most of these results are performed without understanding of biological role of  $^1\text{O}_2$  in the arena of brain hemodynamics and vascular physiology that makes the difficulties in their interpretations and applications in medicine. To appreciate fully the nature of  $^1\text{O}_2$ , it is necessary to understand better the role of  $^1\text{O}_2$  in the cerebral physiology and pathophysiology. Here we are highlighting the current approaches in the fundamental and clinical studies of cerebrovascular effects caused by PDT and generation of  $^1\text{O}_2$ , focusing mainly on the analysis of mechanisms and promising practical applications.

## 2. Mechanisms of triplet - singlet transition

Oxygen molecule has triplet ground state  $^3\text{O}_2(^3\Sigma_g)$  and the first excited singlet state  $^1\text{O}_2(^1\Delta_g)$  (1273 nm). In the free unperturbed molecule, the transition  $^3\text{O}_2 \rightarrow ^1\text{O}_2$  is forbidden as electric-dipole transition due to the even parity of the ground and excited states. Theoretical and experimental data give transition rate of spontaneous emission for isolated oxygen molecules  $2.3 \cdot 10^{-4} \text{ s}^{-1}$  that corresponds to long radiative lifetime of 72 min [11].

Two mechanisms of generation of singlet oxygen by laser irradiation were experimentally established in tissues and cells. The first one is the formation of singlet oxygen via the photodynamic process which involves energy transfer to oxygen from singlet and triplet excited states of photosensitizer (PS) molecule [12] (see Fig. 1 A). The second mechanism is related to a direct oxygen photoactivation by 1270 nm laser irradiation [13] (see Fig. 1B). However, the direct transition  $^3\text{O}_2 \rightarrow ^1\text{O}_2$  is forbidden for unperturbed oxygen molecules, at the high oxygen concentration in gas phase and in the condensed phase in the presence of other molecules, enhancement of forbidden transition occurs due to collision of  $\text{O}_2$  with surrounding molecules that causes perturbation of the molecular orbitals and the loss of their symmetry. This leads to lifting of the forbidden selective rules and enhancement of this transition. Due to intermolecular perturbation, the transition rate increases up to  $10^4$  times and lifetime of  $^1\text{O}_2$  measured by different techniques is in milli- and microsecond region depending on solvent [11, 13, 14]. These measurements confirmed the enhancement of direct emission and absorption of singlet

oxygen state in solvent according to the mechanism of collision-induced electric-dipole transition of oxygen molecules [11].

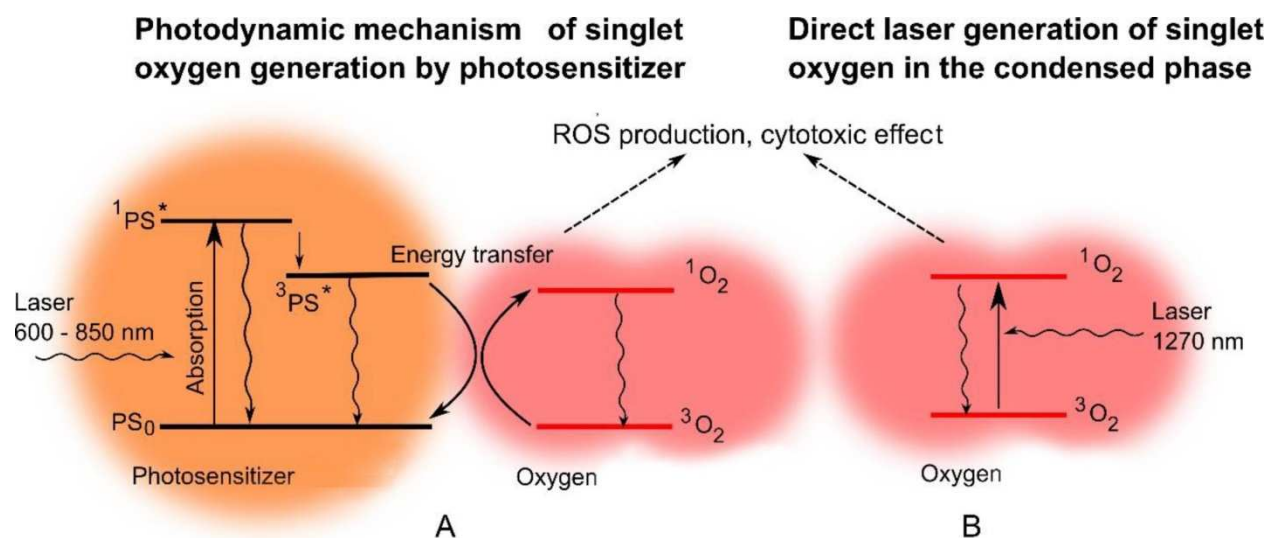


Figure 1. Photodynamic mechanism of singlet oxygen  $^1\text{O}_2$  generation by photosensitizer (PS) through energy transfer to oxygen from singlet and triplet excited states of photosensitizer,  $^1\text{PS}^*$  and  $^3\text{PS}^*$  (A) and direct laser generation of singlet oxygen (B).

Direct photoexcitation of  $^1\text{O}_2$  by 1270 nm laser irradiation has been observed in different liquid media [13-16]. Photosensitizer-free singlet oxygen generation was observed in photo oxygenation of the different traps in various air-saturated solvents and defined the average absorbance  $A_{1270} = 1.33 \cdot 10^{-5}$  at 1270 nm and molar absorption coefficient [13, 15] to the transition  $^3\text{O}_2 \rightarrow ^1\text{O}_2$  in the range of  $(1.5 - 5.1) \cdot 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ . Direct oxygen photoactivation at 1270 nm in inhomogeneous environment was observed in lipid nanocapsules dispersed in water using the water and non-water soluble chemical traps [17]. The experiment in heterogeneous media that mimics biochemical environment can provide a promising method to understand singlet oxygen dynamics in living cells. Development of quantum-dot (QD) laser diodes (LDs) emitting in the near infrared (NIR) spectral range has opened up new venues in low-intensity laser therapy. The QD-LD emission wavelength centred at around 1268 nm coincides well with the near IR absorption band [1, 5] of oxygen molecule (Fig. 2A). One particular opportunity involves activation of the apoptotic response through direct molecular oxygen photoexcitation. Furthermore, the action spectra of a number of cell cultures, recorded in the spectral range from 310 to 860 nm, and the results demonstrated for low-intensity laser therapy [8] suggest transformation of cell metabolism in response to low power laser excitation in

the spectral intervals consistent with absorption bands of molecular oxygen.  $^1\text{O}_2$  formation by direct photoexcitation with 1265 nm in pigment-free aerobic systems [1, 5] and in condensed phase at 77 K with 1064 nm [9] have also been demonstrated. Furthermore direct induction of singlet oxygen by laser irradiation at 1270 nm in different cell cultures was reported by several research groups and its effect on cellular processes such as oxidative stress, DNA damage, and apoptosis was investigated in cancer cells [10, 18, 19].

### *2.1. Singlet oxygen generation in organic solution.*

To investigate direct photo-activation of molecular oxygen we used a fibre coupled InGaAs/InAs quantum dot laser diode (Innolume GmbH) in continuous wave regime and emission spectrum centred at 1268 nm as an irradiation source (for experiments below) and a well known scavenger of singlet oxygen (naphthacene in carbon tetrachloride) as a substrate for the photo-oxygenation assessments [20]. The concentration of the  $^1\text{O}_2$  naphthacene in solutions used did not exceed 200  $\mu\text{M}$  in order to avoid possible concentration quenching. The difference in absorption at a given wavelength before and after laser irradiation was used as a measure of the  $^1\text{O}_2$  formation ratio. The interaction of this compound with  $^1\text{O}_2$  is known to be purely chemical, accompanied by formation of endoperoxides and loss of absorbance in the visible spectral range. The absorption spectrum of naphthacene shows no resolvable absorption at 1268 nm (data not shown) that avoids doubts on whether the laser pulse can directly bleach naphthacene. The 20-min 1268 nm irradiation led to appreciable bleaching of the air-saturated solutions with a twofold increase in the bleaching rate in oxygen-purified solutions at an irradiation dose of 1200  $\text{J}/\text{cm}^2$ . Control 830 nm LD pulse induced no detectable changes (within a measurement error of 2%) of naphthacene absorption even in oxygen-enriched solutions. The absorption spectra of a control sample before and after 830-nm laser irradiation and a negative control as a measure of photobleaching under normal room lighting conditions are shown in Fig. 2B. Oxidation of naphthacene was determined by the  $^1\text{O}_2$  generation due to direct 1268 nm laser  $^3\text{O}_2$  photoexcitation and suggested that similar photo-oxidation reactions might be detected in living cellular systems.



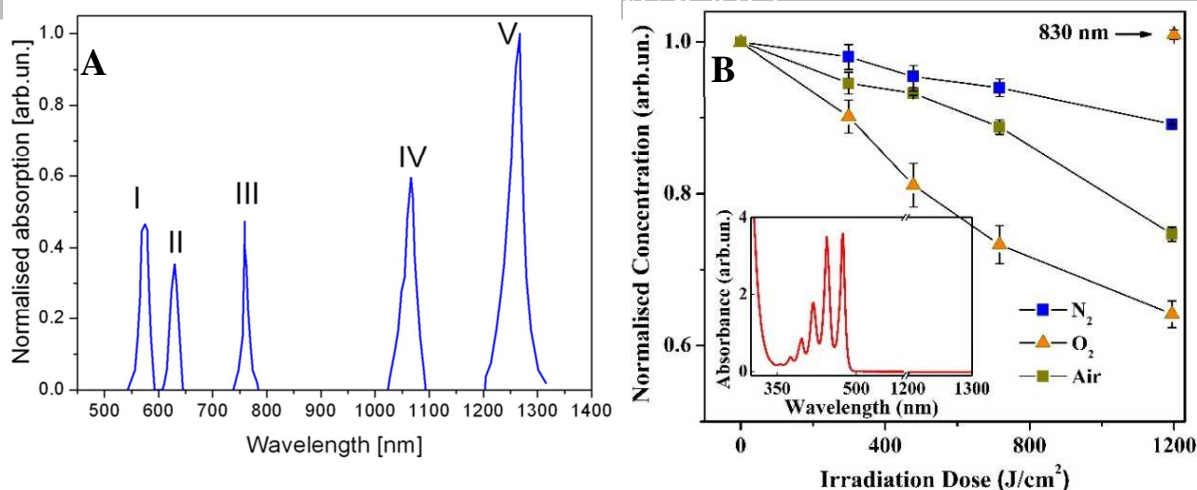


Figure 2.(A) – Absorption spectrum of molecular oxygen in gaseous phase by [1, 5]. (B) – Photobleaching of naphthacene in CCl<sub>4</sub> solution by 1268 nm laser irradiations at different gas perfusion. *Insertion*: Naphthacene absorbance spectrum.

## 2.2. Singlet oxygen generation in living cells

Laser-induced <sup>1</sup>O<sub>2</sub> production in living cells. The feasibility of oxygen photo-activation in the absence of PS in true living cell systems remains highly uncertain. However the results of low-intensity laser therapy [7, 8, 21], modification of red blood cell membrane proteins [22], and cancer cell growth suppression in PS free-conditions [10] by photo-excitation in the near infrared spectral range suggests direct photo-oxidation in media containing molecular oxygen. Since the feasibility of oxygen photo-activation in the absence of PSs in true bio-systems is still under doubts, we chose dihydroethidium (DHE) which is specifically oxidized to dihydroxyethidium (DHOE) fluorescing at 585 nm [23] by the superoxide anion (O<sub>2</sub><sup>•−</sup>, the first by-product of <sup>1</sup>O<sub>2</sub> reduction [24] and ROS precursor in the cell [23]) to monitor singlet oxygen amount in modified immortal skin keratinocytes (HaCaT) [25] before, during, and after laser pulse irradiation.

HaCaT cells show a significant deference between non-irradiated cells and those irradiated by 1268 nm laser pulse of fluence 47.7 J/cm<sup>2</sup> that caused an increase in DHOE fluorescence with a lag-phase of 40-60 s reaching the steady-state level in 5 min and continued even 3 min after the termination of the laser application (Fig. 3A). Experiments using HaCaT cells showed a significant deference between non-irradiated cells and those irradiated by a 1268 nm laser pulse of 47.7 J/cm<sup>2</sup> causing an increase in dihydroxyethidium (DHOE, oxidized DHE) fluorescence reaching a steady-state level after 8 min and continued post termination of the laser pulse (Fig 3). At the same time, a strong donor of O<sub>2</sub><sup>•−</sup> NaOCl (100 μM) induced a dramatic increase in DHOE fluorescence. The pre-incubation of HaCaT cells for 10 min with α-tocopherol (nonspecific ROS scavenger [26,



27)) diminished the laser-induced fluorescence to near background levels. This suggests that the 1268 nm laser irradiation photo-oxidizes triplet molecular oxygen inside the cell.

Different methods of singlet molecular oxygen detection in tissues including a time resolved luminescence spectroscopy and luminescence lifetime measurements at 1268 nm were overviewed recently [28].

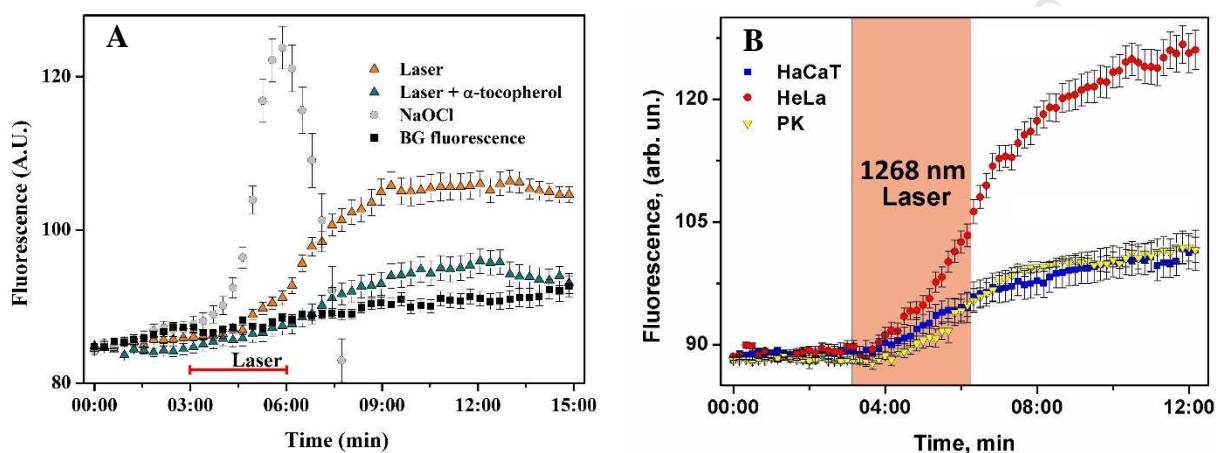


Figure 3. 1268 nm QD laser irradiation induced singlet oxygen generation in HaCaT (A, B), primary keratinocytes (PK, B), and HeLa cells (B)

Primary keratinocytes (PK), HaCaT and HeLa cell lines were investigated for ROS response to 1268 nm laser irradiation. As shown in figure 3 (A and B) the 1268 nm laser pulse triggered  $O_2^-$ -dependent fluorescence in all three cell lines with the most dramatic effect observed in HeLa cells and no difference between HaCaT and PK. The NIR laser-induced fluorescence demonstrated strong dose dependency without reaching saturation with the time, especially in HeLa cells (Fig. 3B). The increased sensitivity of HeLa cells could be explained by their malignant origin resulting in a high metabolic state leading to a weaker free radical defense system [29] compared with noncancerous cells [30]. A LD emitting at 830 nm was employed as a temperature control as nearly identical heating was evident compared with 1268 nm LD.

The 830 nm irradiation having no absorption by  $O_2$  shows no effect on  $O_2^-$ -dependent fluorescence in any cell types [31] underpinning the hypothesis that 1268 nm LD irradiation can induce ROS production through singlet oxygen photoactivation rather than by heat. Most intriguing was the observation of a continued increase in ROS level inside the cells even after the laser had ceased, most prominent in HeLa cancer cells.

### 2.3. Cytosolic free calcium level and ion channel activity under laser pulse

Products of oxidative stress (ROS, NO, organic radicals, etc.) are recognized as powerful regulatory messengers in cell signaling which very often affect cell calcium homeostasis [32]. In turn calcium homeostasis disruption can contribute to oxidative stress [33]. From our results, we anticipated that laser-induced  $^1\text{O}_2$  production could reflect on cytosolic calcium concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ). Therefore single cell ratiometric  $\text{Ca}_2^+$  imaging was employed to estimate calcium response of the HaCaT and HeLa cell lines after 1268 nm irradiation of  $47.7 \text{ J/cm}^2$  (Fig. 4A).

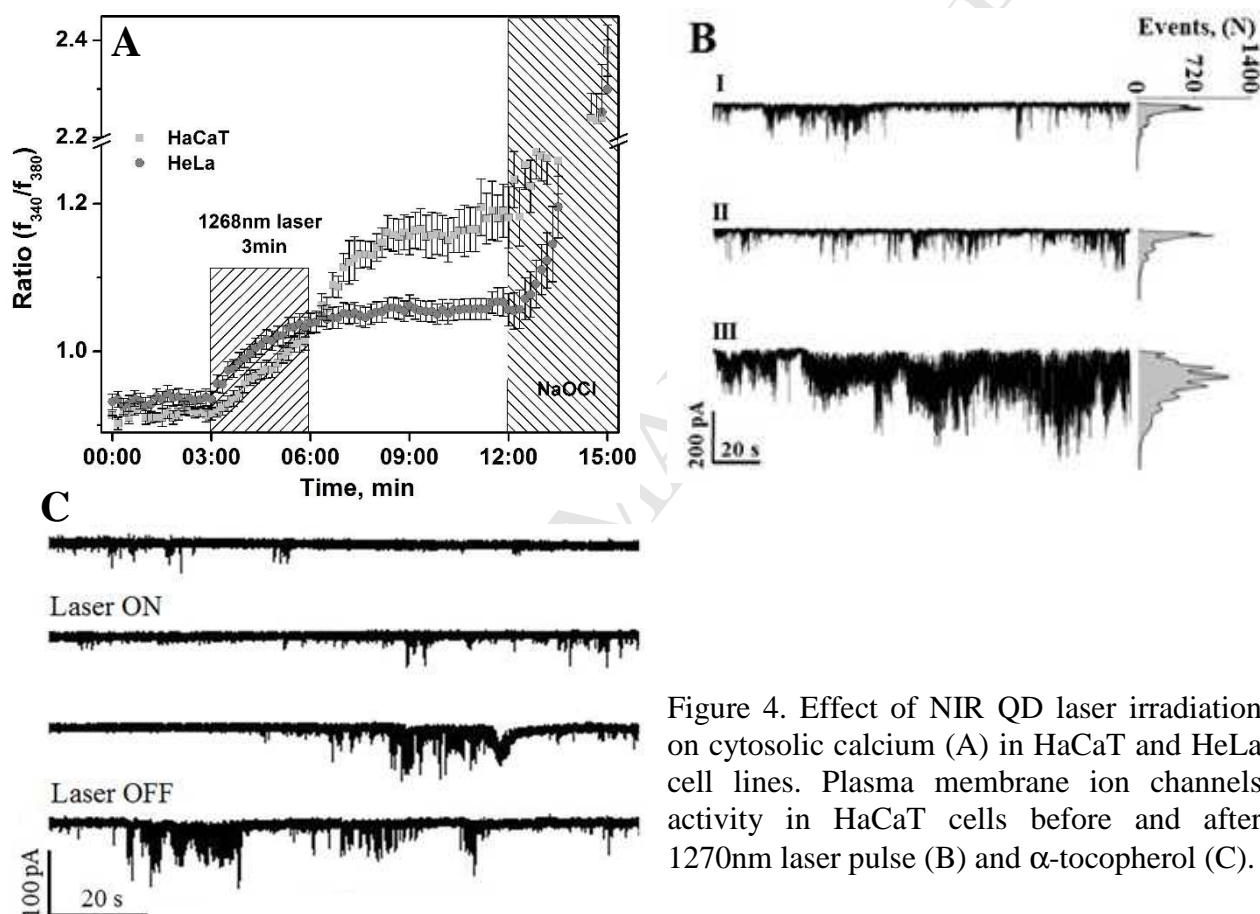


Figure 4. Effect of NIR QD laser irradiation on cytosolic calcium (A) in HaCaT and HeLa cell lines. Plasma membrane ion channels activity in HaCaT cells before and after 1270nm laser pulse (B) and  $\alpha$ -tocopherol (C).

Imaging showed an apparent increase in the fluorescence ratio by more than 1.2 times for HaCaT and HeLa cells, registered immediately after the laser was on. Following the cessation of irradiation,  $[\text{Ca}^{2+}]_{\text{cyt}}$  was measured for at least 7 min and found to continue rising in HaCaT cells whilst HeLa cells demonstrated plateau being opposite the laser-induced singlet oxygen response. Further application of NaOCl ( $100 \mu\text{M}$ ) as a positive control induced typical oxidative-stress-like calcium response in all cell lines (Fig. 4A). The LD emitting at 830 nm also temporally increased calcium fluorescence in both cell types falling to basic levels after the pulse terminated (data not shown).

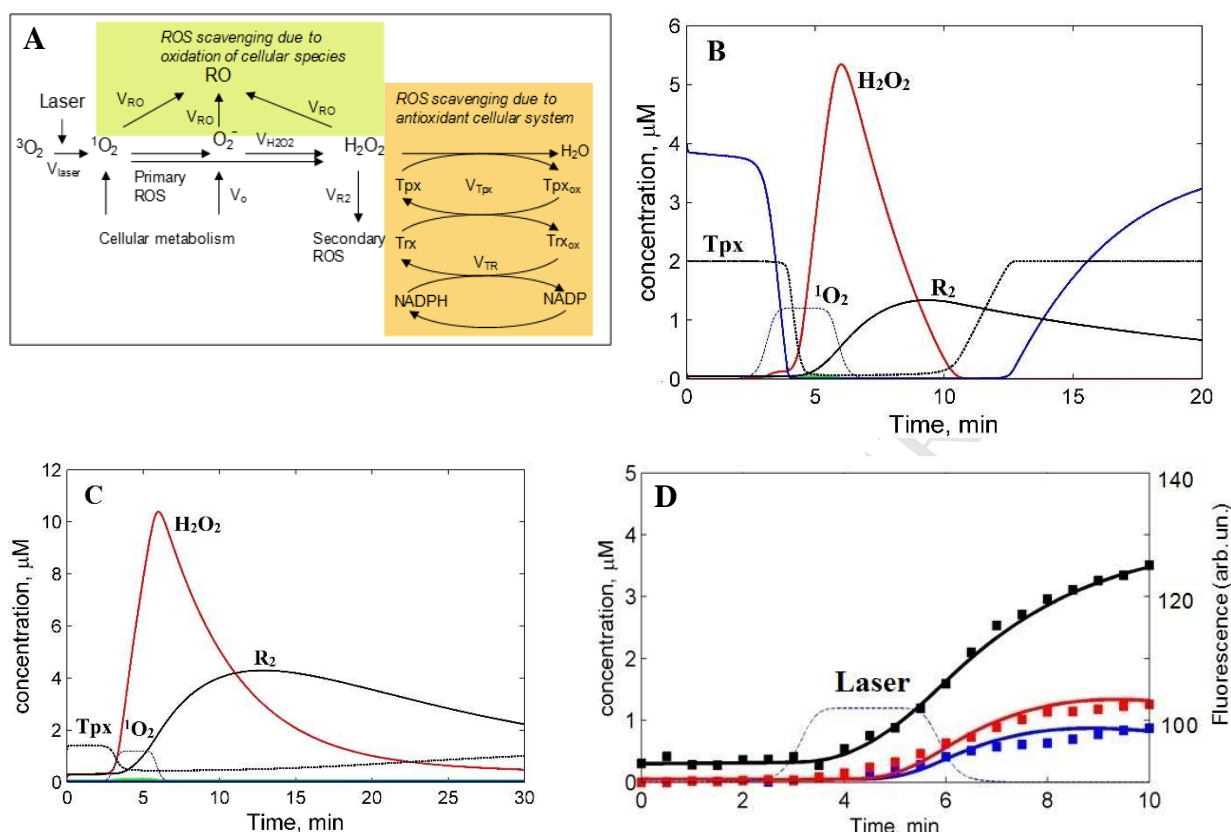
#### 2.4. Modelling Laser induced ROS production in different cell lines

To answer where NIR irradiation-induced calcium originates from external or internal calcium stores we monitored single channel activities (Fig 4B, traces) on the plasma membrane of HaCaT cells with a patch clamp in a cell-attached configuration before (I), during (II) and after (III) an NIR pulse. The analysis of single-channel activity demonstrated a typical pattern (values of current and time of a single open event) associated with non-excited tissue low-voltage activated  $\text{Ca}^{2+}$  channels [34]. Neither amplitude nor the number of open events changed during 3 min laser irradiation with immediate activation of the channels after irradiation was ceased, demonstrating a rise in the number of open events by more than an order of magnitude (Fig. 4B, III). Pre-incubation with  $\alpha$ -tocopherol (10  $\mu\text{M}$ ) for 10 min decreased general channels activity and fully prevented NIR-induced channel activation (Fig. 4C) pointing to ROS origin of the laser effect on the channel activity. Thus suggesting that the observed increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$ ,  $\text{Ca}_2^+$  channels activity and  $\text{O}_2^-$ -production in the cell is likely to be associated with direct molecular oxygen photoactivation by 1268 nm irradiation. Cancerous HeLa cells demonstrating highest  $\text{O}_2^-$  production in response to the laser pulse being more vulnerable compared to noncancerous cells due to a higher general metabolic activity at the same time logically showed weaker calcium response as a protection means [35, 36].

For comprehensive analyse of our results in terms of oxidative stress we developed a kinetic model of redox homeostasis and its imbalance by laser-induced  $^1\text{O}_2$  generation (Fig. 5). The model takes into account (Fig. 5 (A, B and C)): (i) generation of endogenous ROS ( $\text{O}_2^-$ ) and laser-induced  $^1\text{O}_2$ ; (ii) their dismutating into  $\text{H}_2\text{O}_2$  by superoxide dismutase (SOD) and non-enzymatically; (iii) generation of secondary pool of ROS from  $\text{H}_2\text{O}_2$ ; and (iv) scavenging ROS by the thioredoxin peroxidase/thioredoxin/thioredoxin reductase and glutathione (GSH) antioxidant system (Tpx/Trx/TR/GSH) which protects the cell from most types of oxidative stresses [37]. The model describes the first stage of oxidative stress and antioxidant cellular response to it during the first 5 min after the laser is off.

The mechanism of secondary growth of ROS after the laser pulse is determined by excessive production of  $\text{H}_2\text{O}_2$  from laser-generated  $^1\text{O}_2$  via its conversion into  $\text{O}_2^-$ . Prolonged retention of a high level of  $\text{H}_2\text{O}_2$  results in depletion of the scavenging Tpx/Trx/TR/GSH system and its slow restoration after laser irradiation (Fig 5D, blue plot). Assuming that laser-induced  $^1\text{O}_2$  production rate,  $V_{\text{laser}}$  was proportional to the irradiation dose we calculated ROS kinetics at different  $V_{\text{laser}}$  values and compared these

results with experimental data at different irradiation doses in HaCaT cells (Fig. 5D, blue and red plots).



**Figure 5.**

The scheme of the model describing cell redox homeostasis and its imbalance as result of ROS generated by laser pulse (A). (B) in normal and (C) cancerous cells.  $H_2O_2$  (—); reduced Trx (—); primary ROS ( $^1O_2$  and  $O_2^-$ ),  $R_1$  (—); reduced thioredoxin peroxidase, Tpx (—); sum of primary and secondary ROS,  $R_2$  (—); rate of  $^1O_2$  generation by 3 min laser pulse only (---). Kinetics of ROS production in noncancerous cells at the different rates of singlet oxygen generation (D):  $V_{laser,1}$  (—) and  $V_{laser,2} > V_{laser,1}$  (—) and in cancer cells (—). Laser-induced singlet oxygen production rate used for modelling (---). Experimental data of ROS production at 47.7 J/cm<sup>2</sup> (■) and 71.6 J/cm<sup>2</sup> (■) in HaCaT and 47.7 J/cm<sup>2</sup> in HeLa (■) cells. The model describes “primary” ROS generation [34] due to superoxide anion radical ( $O_2^-$ ) production ( $V_o$ ) as a result of mitochondria function and NADPH oxidase catalysis and  $^1O_2$  ( $V_{laser}$ ) generation by 1268nm laser pulse. “Primary” ROS is rapidly dismutated into  $H_2O_2$  by superoxide dismutase, SOD, and non-enzymatic reaction ( $V_{H2O2}$ ) [34]. The model considers two ROS scavenging systems: (i) the Tpx/Trx/TR cascade converting  $H_2O_2$  to  $H_2O$  ( $V_{Tpx}$  and  $V_{Trx}$ ) by oxidizing Tpx and Trx and with their further reduction by TR ( $V_{TR}$ ); and (ii) due to oxidation of cellular biomolecules ( $V_{RO}$ ). The “secondary” ROS (the hydroxyl  $\cdot OH$ , peroxyl  $ROO\cdot$ , and others radicals) are generated from  $H_2O_2$  ( $V_{R2}$ ) by Fenton reaction [37].

To model relatively high ROS generation observed in cancer cells than in noncancerous cells (Fig. 5B, C) we have changed parameters to consider the key features of ROS metabolism in cancer cells i.e a higher rate of general ROS production [38] ( $V_o$ )

and higher expression of Tpx, Trx, and TR [39, 40]. The ROS kinetics computed with these parameters closely mimicked high ROS accumulation in cancer than in noncancerous (Fig. 5D, black plot) which is in reconciliation with experimental data for HeLa cells (Fig. 3). Concluding the higher laser-induced ROS level observed in cancer cells might result from the depletion in reduced Tpx/Trx/TR/GSH caused by high general ROS production (Fig. 5B, C) involving cell calcium homeostasis disruption and could be a trigger for apoptosis. Note, a significant depletion in intracellular GSH level as a result of cell exposed to 1265 laser was observed in cancer cells HCT-116 in comparison to normal CHO-K cells [37]. Theoretical and experimental results lead to conclusion that cancer cells are more vulnerable to 1265 laser exposure than normal cells. Finally, these findings propose a new tumour therapeutic approach based on direct 1268 nm laser photoactivation of molecular oxygen.

### 3. The role of singlet oxygen in cerebrovascular physiology

#### 3.1. The singlet oxygen-induced thrombotic stroke: different interpretation of coagulation mechanisms *in vivo* and *in vitro*

Stroke, also known as brain attack or cerebrovascular catastrophe, is a disease that affects the blood vessels that supply blood to the brain. From the beginning of the 21 century, stroke has become the leading causes of death and disability worldwide [57]. In 1999, stroke was responsible for 5.5 million deaths worldwide [58]; according to forecasts, the incidence of stroke without adequate therapy will increase to 6.3 million deaths in 2015 and 7.8 million in 2030 [59]. Despite the fact that stroke is most common “killer” of man and very important socio-economic problem, very few therapeutic measures and drugs are available to treat this disease [60,61]. The use of animal models in recent years has provided a better understanding of the nature of stroke [62].

The thrombosis of cerebral vessels is a serious complication of stroke with the incidence range from 10 to 75%, depending on diagnostic methods and time of evaluation [63]. To develop the new strategies in antithrombotic therapy and effective prevention of stroke, the mechanisms of thrombotic stroke are actively studied in the pre-clinical investigations. To mimic clots formation in human with stroke, in 1983 Watson et al. [13] proposed a rat model of singlet-induced stroke in the cortex based on the concept of photothrombosis of Rosenblum and El-Sabban. In 1977, they discovered that small pial vessels in mouse brain could be occluded with aggregated platelets by dye-mediated photochemical reactions [14].



Later this method was largely improved [15-17] but the experimental principle remains the same (Fig. 6). The photothrombotic approach causes a stroke through the photo-activation of intravenous injected light sensitive dye, most frequently used Rose Bengal – disodium tetrachlorotetraiodofluorescein with absorption peak at 562 nm. Other photosensitive dye such as Erythrosine B can be used to produce stroke but this substance is less effective than Rose Bengal for the same concentration [64]. Circulating in blood stream Rose Bengal is bonding to endothelial cell organelles. When dye stained endothelium is illuminated at the appropriate wavelength by a “cold” light source (a green light at 562 nm from a filtered xenon arc lamp or at 532 nm the second harmonic generation from Nd:YAG laser), it releases energy to oxygen molecules. Oxygen ( $O_2$ ) accepts its electronic-state energy and is becoming excited state (singlet) oxygen ( $^1O_2$ ). Singlet oxygen directly interacts with cerebral endothelium peroxidizing lipids and proteins. This causes rapidly (during 40 min) the endothelial damage and specifically stimulates platelet adhesion and aggregation with thrombus formation, which induce local occlusion of pial vessels and acute stroke [15, 13, 17, 64]. The using of singlet oxygen scavenger such as beta-carotene or L-histidine significantly reduce cerebrovascular injuries after photo-activated thrombosis suggesting that stroke develops through singlet oxygen mechanism [65, 66].

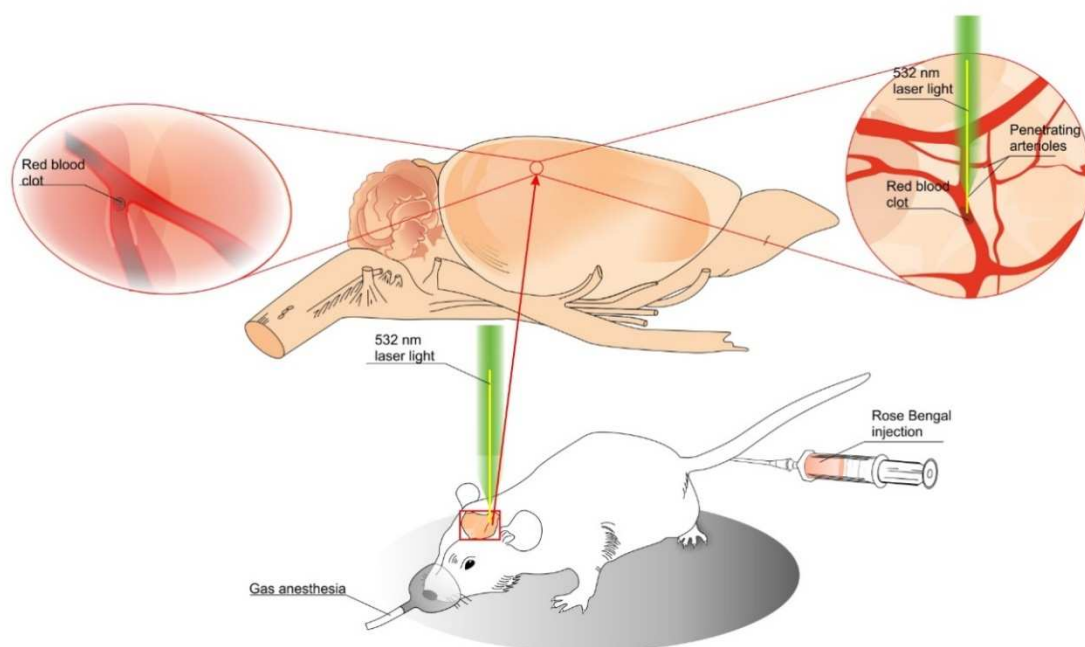


Figure 6. The inducing of thrombotic stroke in rat. The rat underwent to general anesthesia and intravenous injection of light sensitive dye - Rose Bengal, which bonds to cerebral endothelium. Then rat is exposed to “cold” light source (wavelength 532 nm, second



harmonic of Nd:YAG laser radiation), due to dye-mediated photochemical reactions the photosensitizer releases energy to oxygen molecules. Oxygen ( $O_2$ ) accepts its electronic-state energy and is becoming excited state (singlet) oxygen ( $^1O_2$ ). Singlet oxygen directly interacts with cerebral endothelium peroxidizing lipids and proteins. This causes rapidly the endothelial damage and specifically stimulates platelet adhesion and aggregation with thrombus formation.

The advantage of phototrombic stroke model is its non-invasiveness, i.e. it needs only intravenous injection of photosensitizer and light illumination of the intact skull. It does not require complex surgical manipulations such as craniotomy, occlusion of vessels or direct injection of blood into the brain tissues, which are usually used in other models of stroke [62]. The stroke location and degree are predictable, well reproducible and easily controlled by the irradiation intensity and duration, beam position, and dye concentration [67, 68]. Although the mortality rate is less than 10%, phototrombic stroke causes prolonged sensorimotor impairment [69-71].

The criticism of phototrombic stroke model is it does not break only one vessel as it usually happens in human stroke but induces injuries of all superficial small pial vessels due to selectively damages of blood flow in the areas exposed to the light. In order to develop of more clinically relevant stroke model, in 1986 the group of Watson began modification of widely used stroke model as occlusion of middle cerebral artery (MCA) by the local photochemical occlusion of MCA and its branches (frontal, parietal, temporal in the distal-field distribution of MCA [72]. In parallel, they also extended the photochemical method using Rose Bengal for the thrombotic occlusion of the large arteries such as common carotid and femoral arteries. The photochemical occlusion of MCA was modified in other works performing the occlusion of MCA by photo-activation of intravenous infusion of the photosensitizing dye Rose Bengal by 568-nm krypton laser and 355-nm ultraviolet laser irradiation providing more reproducible focal brain injuries [73].

All types of photothrombotic stroke are widely used for the studies of neuroprotective effects of anti-stroke pharmacological agents such as diazepam, a blocker of calcium channels [74], memantine, the antagonist of N-methyl-D-aspartate receptor [75], leptin, the inhibitor of 5' adenosine monophosphate-activated protein kinase [76], melatonin, a hormone of the pineal gland that control sleeping but also reduces the high expression of matrix metalloproteinase 9, which are responsible for opening of blood-brain barrier [77].

Notice that in all above discussed models, authors suggest that the thrombotic effect of  $^1\text{O}_2$  is a main mechanism of stroke in these models. However, in a series of experiments *in vitro* Stief et al. showed direct dose-dependent thrombolysis effect of  $^1\text{O}_2$  [18-20]. Authors mimicked in biochemical experiments the  $^1\text{O}_2$  generation via the activation of polymorphonuclear leukocytes, which are oxidants and chloramine [21]. Using a stable  $^1\text{O}_2$  generator such as chloramine T, platelet-rich plasma and the aggregation agonist (ADP-adenosine-5'-diphosphate or collagen) they monitored the platelet aggregation by the platelet function analyzer. Their results clearly showed that the addition of  $^1\text{O}_2$  release-inducer - chloramine T in concentration 1 mM suppressed 50% of the aggregation capacity of thrombocytes in platelet-rich plasma. Authors also found that the aggregation is reversible to more than 70% by addition of chloramine T in concentration 3 mM. Thus,  $^1\text{O}_2$  can inhibit and reverse platelet aggregation. These results indicate on the direct anticoagulant effect of  $^1\text{O}_2$ . Authors also showed inhibitory effect of  $^1\text{O}_2$  on many processes of hemostasis: fibrinogen, factor V, factor VIII, factor X of human blood [21].

Two described opposite effects of  $^1\text{O}_2$  on the process of coagulation *in vivo* and *in vitro* clearly show to us that we know very little about  $^1\text{O}_2$  nature and its biological significance that makes the challenges or, probably, mistakes in interpretations of  $^1\text{O}_2$ -mediated effects on physiological systems. The further detailed studies are necessary to better understanding of mechanisms responsible for cerebrovascular effects of  $^1\text{O}_2$  with special focusing on the clear detection of  $^1\text{O}_2$  *in vivo*, controlling of  $^1\text{O}_2$  concentration and optimization of oxygenation status.

*The PDT-mediated increase permeability of blood-brain barrier: new strategies in therapy of brain tumour*

Blood brain barrier (BBB) is a “custom” between blood and brain including three functional elements such as astrocytes, neurons and endothelial cells, which selectively restrict passage of various substances to the brain tissues. It protects the brain from foreign entities but also makes challenges for the drugs delivery and limits therapy of many neurological diseases including brain cancer because only few anti-cancer drugs are capable to cross BBB. Among different types of brain cancer gliomas make 80% of all malignant brain tumours and have remained particularly challenging to treat with less than 12-14 months survival after diagnosis.

Although the BBB can be partially disrupted in glioma tissues [78] but the cerebral vessels adjacent to tumour remain intact, i.e. if the tumour mass has a permeable

vascular endothelium, the peripheral brain vasculature has decreased permeability to anti-tumour agents that possesses significant therapeutic challenge. Therefore, the new strategies of increase in the permeability of BBB for ability of the therapeutic agents to reach its targets are crucial step for effective treatment of malignant gliomas. The most popular therapeutic strategies are using of chemotherapeutic agents or ionizing radiation but, unfortunately, neither of these steps had resulted in significant prolongation of life of patients with gliomas.

PDT-related vascular effects of  $^1\text{O}_2$  is potential method to improve drug delivery to the brain due to increase in vascular permeability for pharmacological agents into the local tumour environment (Fig. 7).

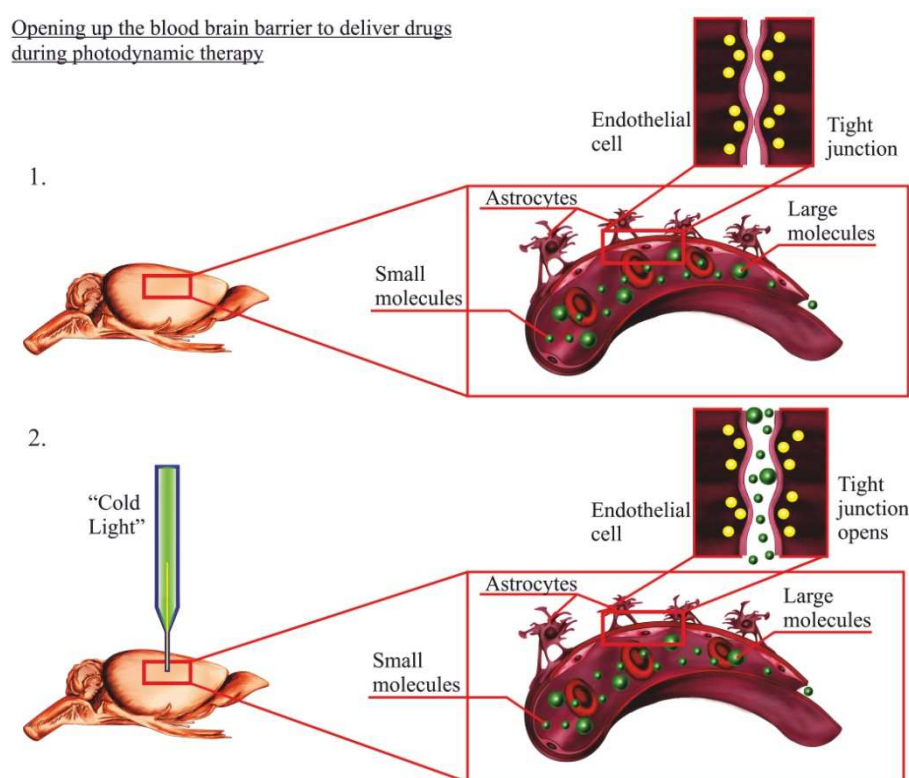


Figure 7. The mechanisms of PDT-mediated BBB disruption. The photosensitizers binds in the cerebral endothelial cells, under light exposure photosensitizers stimulates  $^1\text{O}_2$  generation, which causes the enlargement of endothelial gaps due to changes of the cytoskeleton, cell rounding and constriction via microtubule depolarization [90-93]. 1 – normal state, 2 – the PDT-induced opening of BBB.

Some photosensitizers naturally accumulate in the endothelial cells of vascular tissue allowing 'vascular targeted' PDT. Compared to normal tissues, most types of cancers are

especially active in both the uptake and accumulation of photosensitizing agents, which makes cancers especially vulnerable to PDT. Currently, PDT of brain tumours is used with various photosensitizers (Table 1) [79].

The photosensitizers can have a high affinity for vascular endothelial cells [77, 78]. The traditional explanation of vascular mechanisms underlying PDT of glioma destruction is  $^1\text{O}_2$ -induced damage of the endothelial cells and the vascular basement membrane resulting tumour microvasculature collapse [81, 82]. However, in several studies, it has been shown in several animal models that at low light fluences PDT provided enhanced permeability of BBB with reversible and minimal pathological changes in cerebral vessels [22-25].

Table 1. Photosensitizers applied for the experimental and clinical studies of brain tumours

Photosensitizers	Wavelength, nm	Company	Country
Photofrin	630	QLT Photo Therapeutics	Canada
Photosan	640	AXXO GmbH	Germany
Photohem	630	Moscow State University of Chemical Technology	Russia
Verteporfin	690	Axcan Scandipharm	Norway
Alasens, 5-aminolevulinic acid (5-ALA)	635	State Scientific Center "NIOPIK	Russia
Levulan, 5-aminolevulinic acid, (5-ALA)	400-450	DUSA Pharmaceuticals	USA
Metvix, methyl aminolevulinate, MLA or M-ALA	690	Photocure ASA	Norway
Foscan (m-tatetrahydroxyphenyl (mTHPC)	652	Bio-Litec	UK
Photolon	660	Belmedpreparaty	Republic of Belarus
Photoditazine	650-700	BETA-GRAND	Russia
Al(III) phthalocyanine chloride disulfonic acid - AlPcS2a	670	Frontier Scientific, Inc., Logan, UT	USA

Nowadays, 5-aminolevulinic acid (ALA)-PDT (635 nm, 100 J/cm<sup>2</sup>) is a valuable therapy in the treatment of malignant gliomas because 5-ALA can be orally administered and is relatively safe for application [83-86]. The utility of ALA had been showed in a large (243 patients) multicenter phase III randomized control trials. While 5-ALA itself does not produce fluorescence, after administration into blood or tissue, ALA is metabolized in the cells to protoporphyrin IX that accumulates in tumour tissue giving photodynamic effect via light activation of the target tissue. That allowing neurosurgeon to detect easily and resect accurately tumour and finally to increase 2-fold the survival of patients with a high graded glioma at 6 months [86].

The light sources such as argon-dye lasers, gold vapour lasers, and xenon arc lamps with adequate filters have been used for ALA-PDT. Currently, light emitting diodes and diode lasers are preferable due to their compact size and low cost [87]. Optical fibres are used for light delivery during PDT of gliomas. However, the large size of glioma makes the problems for light delivery and distribution. To achieve this problem, in the novel studies an inflatable balloon filled with a light-diffusing lipid solution are often used. Diffuse light irradiators are important to use for treatment of tumour margins, where glioblastomas grow back in 80% of cases even after complete surgical excision [87-89].

ALA-PDT is also highly effective in temporal opening the BBB in localized region of the brain [83-85]. Hirschberg et al. clearly show that the ALA-PDT causes the opening of BBB in a fluence and fluence rate dependent manner. At the low fluence of 9 J/cm<sup>2</sup>, the ALA-PDT opens the BBB rapidly without any damages of brain tissues, the disrupted BBB is observed during 2 h following PDT and restored during the next 72 h [25]. The disruption of BBB is greater using the higher fluence of 26J/cm<sup>2</sup> but the damages of surrounding tissues (necrosis, edema, hemorrhage) were observed during 17 days.

The mechanisms underlying PDT-mediated opening of BBB remain not fully understood. There are evidences that PDT has direct effect on the capillary endothelial cells [90] inducing the formation or enlargement of endothelial gaps [91] due to changes of the cytoskeleton, cell rounding and constriction via microtubule depolarization [92]. Hu et al. using electronic microscopy observed in the model of C6 glioma (brain tumour cell line) PDT-induced stretching of the tight junction with the increase gaps between endothelial cells [93].

One of promising strategies in anti-glioma therapy is the effects of PDT on the migration of systemically administered exogenous macrophages (Fig. 8). Circulating macrophages have a natural ability to pass the BBB and then undergo differentiation into

'resident' macrophages living long time in microglia [94, 95]. In animal and human biopsies macrophages are often presented in and around glioblastoma multiform [96-98]. One of the reasons for migration of macrophages in glioma is large areas of hypoxia, which is known to change cell behaviour and is associated with extracellular matrix remodelling and increased migratory and metastatic behaviour [99, 100]. Macrophages are attractive to hypoxic regions of tumours and, therefore, they are logical cellular vectors for cancer therapy [22, 101, 102]. For example, by isolating monocytes from a blood of patient, differentiating from blood cells macrophages *in vitro*, loading them with necessary substances (genes, drugs, or nanoparticles) and then re-injecting them into the patient. In the case of gliomas, the use of autologous macrophages have several advantages. The systemically injected macrophages as delivery vehicle could selectively accumulate in specific regions where the BBB has been disrupted. Let's look at one example. The well-known mark of glioma is the loss of heterozygosity on chromosome 10q, which has not a phosphatase and tensin homolog in the PTEN gene playing the important role in the tumour suppression via inducing G1 cell cycle arrest, apoptosis, cell adhesion, migration and differentiation. Despite the promising effects of PTEN gene in cancer therapy, there is significant limitation to deliver the PTEN gene into the brain cells because it cannot crosses the BBB. However, recent various functional studies with using macrophages as transport system for DNA-containing material of PTEN show effectiveness such approaches for activation of anti-cancer effects of PNET in experiments on animals with glioblastoma [113-115].

Notice, the preparation of microphages in vitro and subsequent its re-injection into patients are more easy in comparison to other techniques involving non-circulating cells such as stem cells [103].



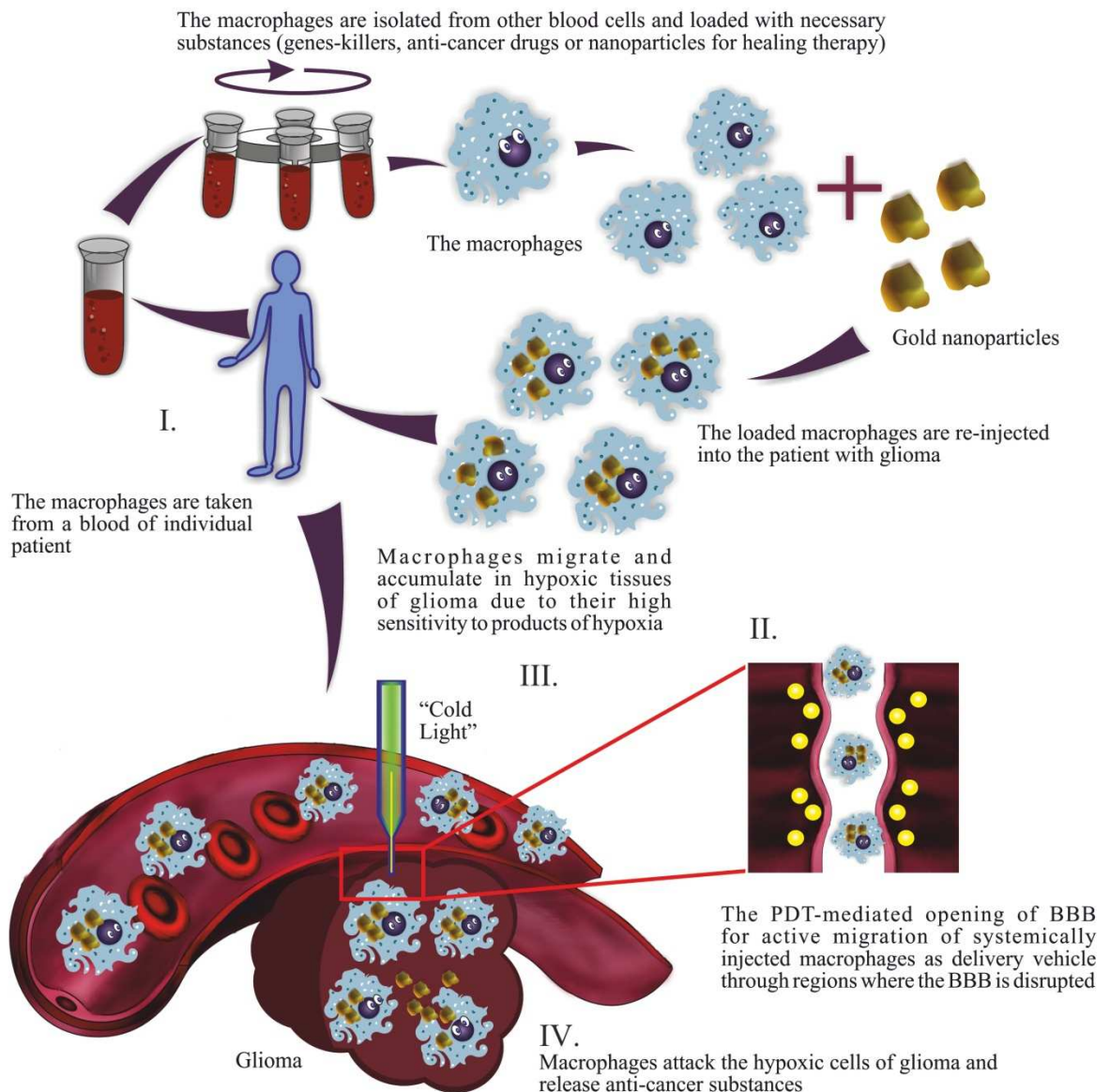


Figure 8. Macrophages as microrobots for glioma therapy. I – The first step: to avoid immune reactions, the macrophages are taken from a blood of individual patient, then they are isolated from other blood cells and loaded with necessary substances (genes-killers, anti-cancer drugs or nanoparticles for healing therapy, see fig. 9), afterwards the loaded macrophages are re-injected into the patient with glioma. II – The second step: it is performed the PDT-mediated opening of BBB for active migration of systemically injected macrophages as delivery vehicle through regions where the BBB is disrupted (see fig. 6). III - Macrophages migrate and accumulate in hypoxic tissues of glioma due to their high sensitivity to products of hypoxia. IV – Macrophages attack the hypoxic cells of glioma and release anti-cancer substances.

Currently, the PDT-induced disruption of the BBB is investigated as a technique for the delivery of therapeutic agents to selective regions of the brain using the migration of

systemically administered exogenous macrophages loaded with iron oxide nanoparticles. The nanoparticles as photoactivated compounds represent emerging photosensitizer carriers and show great promise for PDT [104]. Madsen et al. showed effectiveness of PDT (the photosensitizer AlPcS2a,  $\lambda = 670$  nm; light dose =  $2.5 \text{ J/cm}^2$ ) in the opening of BBB and the migration of exogenous macrophages loaded with iron oxide nanoparticles (120–180 nm) in non-tumour bearing rats[10]. In order to mimic the environment following surgical resection of bulk tumour, where glioma cells have migrated into normal brain adjacent to the tumour site, authors clearly show the extent of loaded macrophages infiltration in rat brain.

Trinidad et al. in experiments *in vitro* using macrophages (Ma) loaded gold nanoparticles (120 nm) and human FaDu cancer cell line (head and neck squamous cell carcinoma) showed the photothermal enhancement of the effects of PDT on the cancer cell viability[31]. They found that the separate application of the photosensitizer AlPcS2a-mediated PDT ( $\lambda = 670$  nm, fluence  $0.25 \text{ J/cm}^2$ ) and photothermal therapies (PTT,  $\lambda = 810$  nm, at irradiance  $14 \text{ W/cm}^2$ ) had little effect on cell viability for the FaDu/Ma (ratio 2:1) hybrid monolayers. However, combination of PDT and PTT at the same laser energy and power settings reduced the cell viability in 2-fold [105].

Using Sprague-Dawley rat glioma model and nanoparticle (120 nm)-loaded macrophage (ratio 4:1) Madsen et al. showed the potential efficacy of PTT for glioma treatment in experiments *in vitro* and *in vivo* [106]. In this case, AlPcS2a-mediated PDT was used as a control treatment to gauge the efficacy of PTT.

The main goal of using PTT is to induce damage of tumour cells due to heating effect with minimizing thermal diffusion to surrounding tissues (Fig. 9).

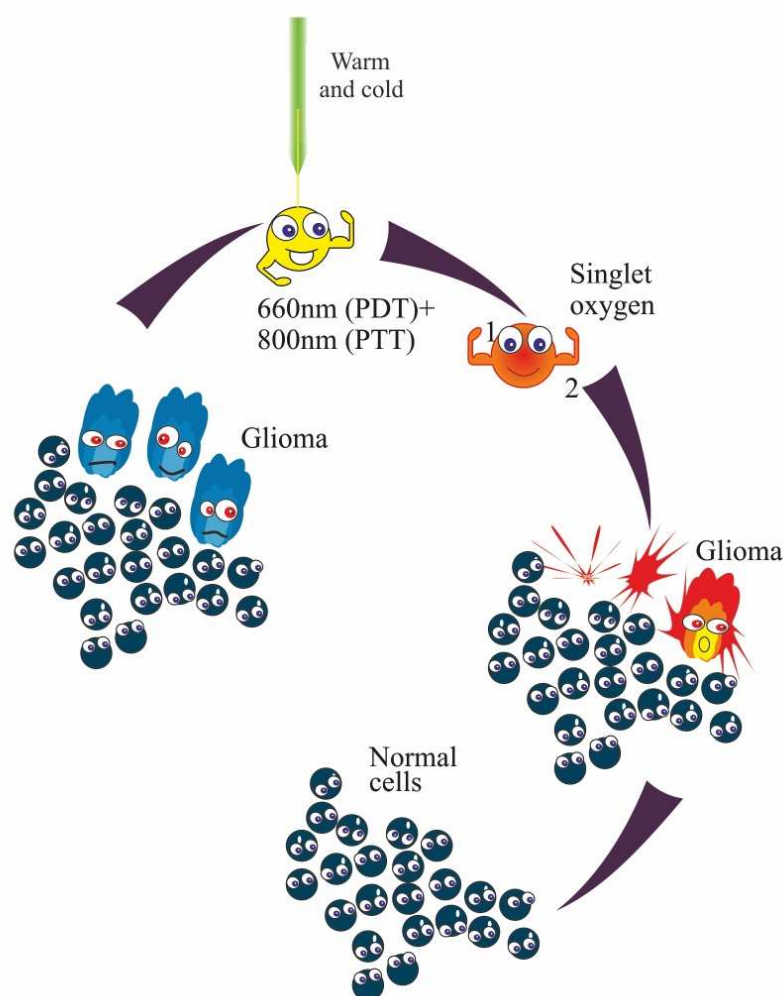


Figure 9. The combination of anti-cancer effects of PDT and PTT. I – The cancer cells growth. II – The PDT-mediated anti-cancer effects (660 nm) include  $^1\text{O}_2$ -induced damage of the endothelial cells and the vascular basement membrane resulting tumour microvasculature collapse [81, 82]. III -The hyperthermic effects of PTT cause the changes on all molecular levels: injuries of cytoskeletal structure, cell membrane rupture, denaturation of protein, impairment of DNA and RNA synthesis, and apoptosis [107]. Also, hyperthermia enhances the oxygen and anti-cancer delivery to cancer cells due to increased blood flow within the heated area [108, 109]. IV – The direct and indirect heating, vascular and cellular effects of PDT- $^1\text{O}_2$  +PTT causes the cancer cells death.

The hyperthermia causes changes on organelle and molecular levels: injuries of cytoskeletal structure, cell membrane rupture, protein denaturation, impairment of DNA and RNA synthesis, followed up by cell apoptosis [107]. The minimum temperature for effective PTT is from 46 to 60°C, but for hypoxic tumour cells or with low pH, the higher temperature elevations may be required [108].

The PTT-mediated enhancement of PDT anti-cancer effect can be related to mechanisms of tumour healing. The tumours have low oxygen and nutrients, high acid concentrations, a disorganized vascular structure that makes cancer cells less able to tolerate the added stress such as heating compared with the normal tissue [109]. Evidently, tumour cells undergo apoptosis as a response to applied heat, while healthy tissue cells can return to a normal state. Hyperthermia increases blood flow within the heated area (2-fold in tumours, 10-fold in normal tissue) [109], that enhances the oxygen delivery to the area [110] giving a synergistic effect of PTT and PDT. As PTT does not require oxygen to provide biological effects, it may be useful for treatment of highly hypoxic tumours such as glioma. Typically, light used for PTT penetrates deeper in tissues compared to light that used for PDT. For example, penetration depth of normal adult brain at 670 nm (PDT) and 810 nm (PTT) is equal approximately to 4.0 and 6.0 mm, respectively [111].

Hyperthermia-induced tumour hyperperfusion also enhances the drug delivery to the cancer cells. For example, moderate PTT has been shown to enhance the effects of chemotherapeutic agents (doxorubicin, bleomycin, cisplatin) on human FaDu squamous cells [112]. The murine macrophages loaded by gold nanoshells (AuNS) combined with cancer cell line were subjected to three cytotoxic drugs with or without near infrared laser irradiation. For all three drugs, efficacy was significantly increased by laser activation of AuNS-loaded Ma.

To improve the delivery of macromolecules and genes into glioma cells in modern studies photochemical internalization (PCI) as a photodynamic therapy-based approach using the light treatment (the photosensitizer AlPcS2a,  $\lambda = 670$  nm) are actively investigated for different lines of glioma cell monolayers or multi-cell tumour spheroids. The utility of PCI for the enhancement of non-vital transfection of suicide genes such a tumour suppressor gene (PTEN) or the cytosine deaminase (CD) into glioma cells and significant decrease in cell survives and growth has been shown in *in vitro* experiments with monolayers of U251 human glioma cells and multi-cell U87 glioma spheroids [113-115].

Collectively, PDT can change the permeability of BBB and might be promising step in progress of anti-gliomas therapy. Although the use PDT for the selectively opening of BBB and delivery of drugs to tumour is attractive, it needs further studies for optimization of PDT condition and tissue oxygenation status, photosensitizer concentration and pharmacokinetics/distribution, scheme of laser illumination and personalized dosimetry (i.e. light, photosensitizer,  $^1\text{O}_2$ ) to avoid vessel damages such as edema, constriction, thrombosis, hemorrhages [116-118]. To assess efficacy of PDT with curative intent, high quality comparative, randomized studies are needed. Palliative treatment with PDT seems to increase the quality of life in otherwise untreatable patients.

*The mechanisms underlying the direct effect of singlet oxygen on vascular homeostasis*

In the previous section, we discussed the effect of  $^1\text{O}_2$  generated by photo-activation on the opening of the BBB via the temporal attack on endothelial cells of cerebral vessels. This new aspect of PDT-induced opening of the BBB is an important novel research direction in the advanced strategies of glioma therapy. However, it is very difficult to explain the nature of effects of  $^1\text{O}_2$  on endothelial cells of cerebral vessels. One of the problems is that there is no direct demonstration of  $^1\text{O}_2$  formation in organism due to its very short lifetime and, therefore, there are no specific traps available to measure  $^1\text{O}_2$  generations *in vivo*. This is a reason why there is very limited information about the direct effects of  $^1\text{O}_2$  on the vessels. The most publications are focused on the indirect evaluation of vascular effects of  $^1\text{O}_2$  using the generally accepted hypothesis and interpretations of vascular effects of PDT [81, 82, 91, 119].

For a better understanding of mechanisms responsible for direct effects of  $^1\text{O}_2$  on the vascular homeostasis, here we focus on the review of a few experiments *in vitro* on the isolated vessels with detection of  $^1\text{O}_2$  by the measurement of  $^1\text{O}_2$ -TEMP product (TEMPO; 2,2,6,6-tetramethylpiperidine-N-oxyl) and the comparison of the similar vascular effects of  $^1\text{O}_2$  in the experiment *in vivo*.

Mizukawa et al. clearly showed that endothelium-dependent vasorelaxation is extremely vulnerable to  $^1\text{O}_2$ . Indeed, prior exposure to photo-activated Rose Bengal of the rings of mesenteric arteries induces irreversible inhibition of the endothelium-dependent relaxation of arterial vessels evoked by acetylcholine and calcium ionophore, but not the endothelium-independent vasorelaxation induced by nitroglycerin [120]. Authors suppose that the loss of endothelial response after  $^1\text{O}_2$  exposure may be due to inhibition of endothelium-derived relaxing factor (EDRF) synthesis but not direct inactivation of EDRF because nitroglycerine also causes vasorelaxation due to activation of EDRF but  $^1\text{O}_2$  did not change vascular sensitivity to nitroglycerine.

The results of experiments *in vitro* confirmed the data obtained *in vivo* with using of splitter-television microscope for visualization of mouse pial arterioles and intravenous injection of photosensitizer such as Evans Blue [121]. Authors demonstrated the  $^1\text{O}_2$ -mediated injury of endothelial-dependent vasorelaxation induced by acetylcholine, bradykinin and calcium ionophore A23197. However, vasodilation by sodium nitroprusside, an independent dilator, was not affected by laser-dye.

In the Hearse's laboratory in a series study of aerobically perfused rat hearts and *in situ* photoactivation of Rose Bengal showed that  $^1\text{O}_2$  directly attacks endothelial cells and



smooth muscles causing vascular incompetence that leads to the progressive impairment of blood flow [4,5]. In these works, authors have demonstrated that photoactivation of Rose Bengal had no effect on heart rate but caused a transient (0-4 min) vasodilation followed by a progressive vasoconstriction that was accompanied by the rapid development of electrocardiographic abnormalities and arrhythmias [122]. Photoactivation by Rose Bengal also resulted in severe ultrastructural damage of endothelial cells such as swelling of mitochondria with disruption and clumping of cristae, the development of contraction band necrosis, extensive degranulation of mast cells, a loss of the calcium with the appearance of intramitochondrial calcium precipitates while in the normal state the calcium is localized in the inner sarcolemmal surface [123].

The  $^1\text{O}_2$ -related injuries of calcium homeostasis causes the disorders of adrenergic mechanisms of vasoconstriction that strongly depend on the concentration of calcium ions.

Yoshino et al. studied the direct effects of  $^1\text{O}_2$  on adrenergic neurotransmission using a high performance liquid chromatographic procedure. They showed that exposure of helical strips of rabbit mesenteric vein to  $^1\text{O}_2$  is accompanied by a significant increase in noradrenaline (NA) release [124]. Mizukawa et al. studied the direct effect of  $^1\text{O}_2$  on the constriction of the ring of rabbit mesenteric artery induced by NA [120]. They found that NA-related constriction was attenuated by previous exposure of arterial vessel to photolysed Rose Bengal. These changes were without a significant reduction in maximum tension generation suggesting that receptor dysfunction may be involved in this vascular effect of  $^1\text{O}_2$ .

Thus, two experiments clearly show that  $^1\text{O}_2$  induces the NA release in synaptic cleft and, in the same time,  $^1\text{O}_2$  depresses NA-mediated constriction without the reduction of vascular wall tension. It is highly likely that  $^1\text{O}_2$  indeed causes the increase in the NA release in synaptic cleft but, in parallel,  $^1\text{O}_2$  “blocks” the alpha-adrenoreceptors on postjunctional side. This means that NA as neurotransmitter even being in high concentration in synaptic cleft cannot cause vasoconstriction due to the loss of his targets, i.e. adrenoreceptors.

The photoactivation of Rose Bengal leads not only to the generation of  $^1\text{O}_2$  but also super anion, hydroxyl radical, hydrogen peroxide, which might impose an oxidant stress and induce the vascular endothelial dysfunction. The direct vascular effect of  $^1\text{O}_2$  was confirmed in the experiments with using of a variety of scavengers (superoxide dismutase, catalase, 1,3-dimethyl-2-thiourea), which eliminate the effects of oxygen-derived free radicals such as super anion, hydroxyl radical, hydrogen peroxide and histidine – a  $^1\text{O}_2$  quencher. Mizukawa et al. clearly showed that only histidine has effects on the photoactivated changes in the vascular endothelial and adrenergic regulatory mechanisms. Authors believe that  $^1\text{O}_2$  rather than other oxygen-derived free radicals responsible for the vascular dysfunction [119].



The cerebral oxygenation is a major regulatory factor, which significantly modulates the cerebral circulation and tone of brain vessels [https://www.ncbi.nlm.nih.gov/books/NBK53082/]. Irradiation of brain in humans by 1064 nm-laser, stimulates the increasing of cerebral oxygenation that is accompanied by improving of cognitive and emotional functions [125-127]. Increase of cerebral oxygenation could be due to generation of  $^1\text{O}_2$  as 1064 nm-laser radiation is efficient for that (Fig. 2A). Therefore, we may expect potentially indirect effects of  $^1\text{O}_2$  on cerebral circulation via improving of metabolic processes. Our hypothesis is based on the experimental data of Tian F. et al. [126], who show the results of the cerebral hemodynamic responses in healthy volunteers to the 1064 nm laser stimulation in the centre of the forehead at the medial frontal lobes bilaterally and at the right lateral frontal lobe, respectively (Fig. 10, experiment I and II). In both experiments, transcranial laser stimulation induced an increase of oxygenated haemoglobin concentration and a decrease of deoxygenated haemoglobin concentration from the baseline in both cerebral hemispheres. The haemoglobin concentration demonstrated an increase associated with the laser stimulation. Human head tissues including skull is rather penetrative for laser radiation of the wavelengths corresponding to the absorption bands of molecular oxygen spectrum (Figure 2.(A)) [127-129].

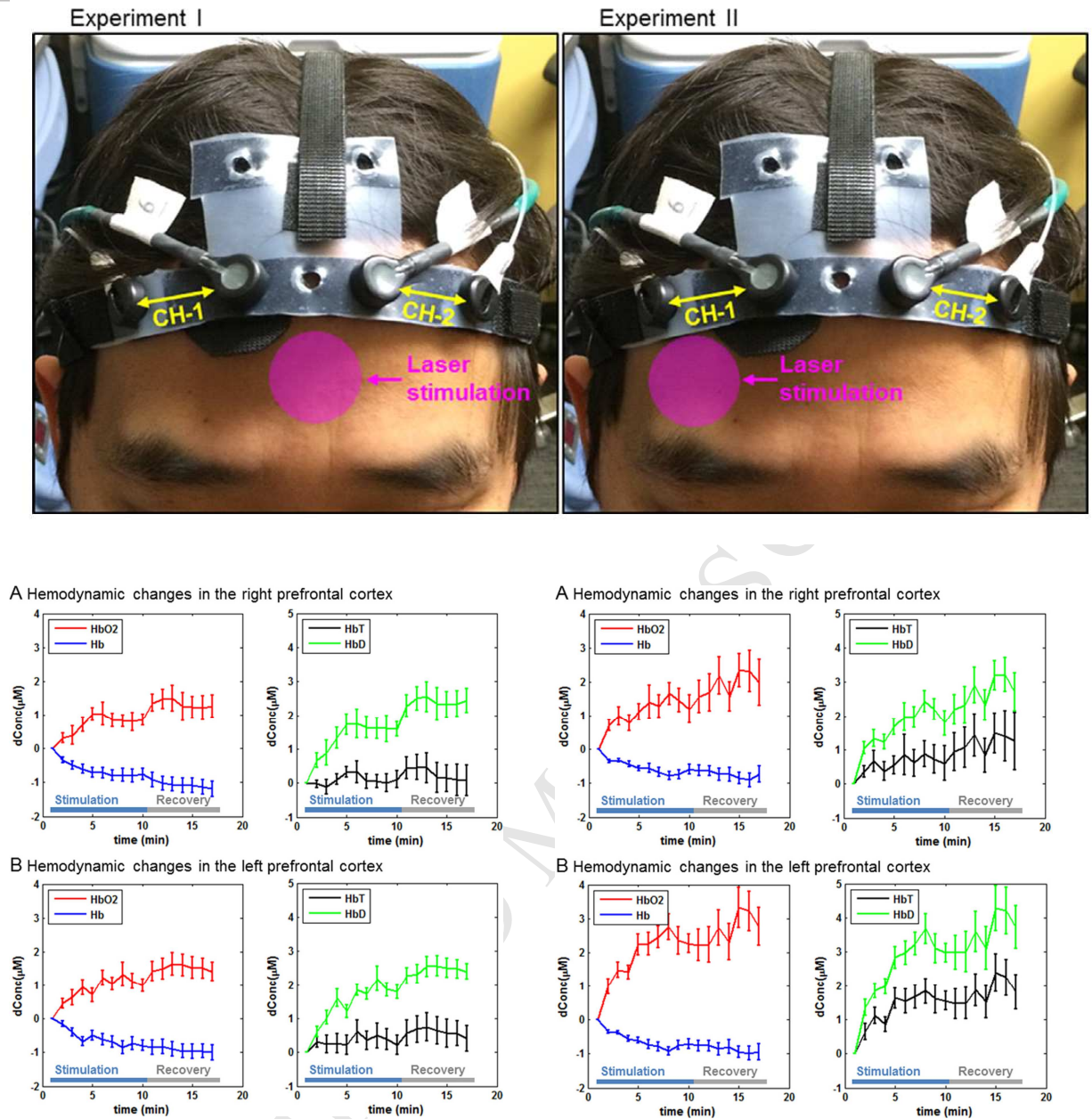


Figure 10. Transcranial laser stimulation: positions of the laser stimulation (4.16-cm diameter of the shined area, the source-detector separation was 3.5 cm for each channel) and configuration of the functional near-infrared spectroscopy (fNIRS) probe in experiment I, and experiment II [126]. The fNIRS probe consisted of two measurement channels (CH-1 and CH-2), one channel over each cerebral hemisphere. Prefrontal hemodynamic responses (mean  $\pm$ SE) in the first experiment: hemodynamic changes in the right prefrontal cortex (A) and hemodynamic changes in the left prefrontal cortex (B). Prefrontal hemodynamic responses (mean  $\pm$ SE) in the second experiment: hemodynamic changes in the right prefrontal cortex (A) and hemodynamic changes in the left prefrontal cortex (B).

Collectively, the analysis of studies of direct vascular effect of  $^1\text{O}_2$  clearly show that imbalance of endothelial regulation of vascular relaxation are an important mechanisms underlying the decreasing of vascular resistance to photoactivated oxidative stress. In these mechanisms, the injury of calcium homeostasis (the redistribution of calcium deposition, reduction of influx and release of calcium from intracellular stores) plays the essential role. This in turn leads to the disorders of adrenergic mechanisms of vasoconstriction that strongly depend on the concentration of calcium ions. These data suggest that  $^1\text{O}_2$ -mediated injuries of calcium resource for vasoconstriction and vasorelaxation are the crucial elements of mechanisms underlying direct vascular effects of  $^1\text{O}_2$ . However, direct generation of  $^1\text{O}_2$  in brain of humans may have positive effects on brain metabolism that can be a new informative platform for better understanding of cerebrovascular effects of  $^1\text{O}_2$ .

## Conclusions and outlook

In this review, we made an attempt to outline the recent advances in using lasers for the generation of singlet oxygen, and have presented promising applications of NIR lasers use for cancer therapy.

Use of photosensitisers to generate singlet oxygen ( $^1\text{O}_2$ ) against cancer opened the door for the introduction of photodynamic therapy (PDT) into practical medicine. Nowadays, PDT is widely used in clinics to treat many pathological conditions, including skin and neurodegenerative diseases. Nevertheless, due to several important limitations of PDT, such as low tissue penetration of activating light (630 nm), high toxicity of photosensitisers and their low specificity to cancer type, the widespread clinical use of PDT is hindered.

Here we showed a new "gold key" to overcome some of the limitations of PDT, such as low light penetration and high toxicity of photosensitizers, by direct  $^1\text{O}_2$  generation using quantum-dot laser diodes emitting in the near infrared spectral range (1268 nm). Regardless of what the pioneering *in vitro* experimental studies suggest about the effectiveness of such an approach for cancer therapy, it is extremely necessary to do further detailed investigations to achieve a fundamental understanding of the mechanisms underlying biophysical properties of direct  $^1\text{O}_2$  generation and its medical significance. For example, it is unclear how to explain the direct  $^1\text{O}_2$  generation because the straightforward  $^3\text{O}_2 \rightarrow ^1\text{O}_2$  transition in molecular oxygen is prevented by the spin-orbital selection rules. Furthermore, the physiological effects of directly generated  $^1\text{O}_2$  are not well explored, strongly requiring new experimental studies to

answer the question: “Whether the laser directly photoactivates molecular oxygen in true bio-systems or whether this is an effect of extensive heating?”

The cerebrovascular effects of  $^1\text{O}_2$  are the highly perspective for the urgent medical application of PDT. Over the last two decades, intriguing new results on cerebrovascular effects of  $^1\text{O}_2$  generated during PDT have been shown. However, this attractive use of PDT in clinical practice strongly needs optimization and adaptation due to negative complications such perivascular oedema, constriction of brain vessels, thrombosis, small cerebral haemorrhages. To overcome successfully these limitations, further studies need to answer the questions: “What are the optimal photosensitiser concentration and pharmacokinetics for the safe and reversible opening of the BBB? What kind of schemes for laser illumination and personalised dosimetry should be avoided to prevent cerebral vessel damages using PDT?”

Another application for the cerebrovascular effects of  $^1\text{O}_2$  is a rodent model of singlet-oxygen induced stroke. However, even though this model has already been employed for 33 years to conduct pre-clinical studies of neuroprotective effects of anti-stroke pharmacological agents, the mechanisms of vascular damage effect of  $^1\text{O}_2$  are not clear. For instance, the thrombotic effects of  $^1\text{O}_2$ , which are usually interpreted as a main cerebrovascular effect of  $^1\text{O}_2$ , were not confirmed in experiments *in vitro* with direct evaluation of  $^1\text{O}_2$  generation and influences of  $^1\text{O}_2$  on the coagulation processes.

Thus, the direct generation of  $^1\text{O}_2$  free of use of exogenous photosensitizers and new promising perspectives of cerebrovascular effects of  $^1\text{O}_2$  with deep and detailed understanding of  $^1\text{O}_2$  nature and its biological significance, will shed light on the new horizons of improving clinical application of laser based therapy for many diseases of the central nervous system as well as give a new impulse for progress in the pharmacological industry of neuroprotective drugs due to a “new gold key” into the brain.

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